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PATENT
Docket No. GC396-2

MODIFIED FORMS OF PULLULANASE

Related Applications

The present application is a continuation-in-part application of United States Application Serial Number 09/034,630 filed March 4, 1998 which is incorporated herein in its entirety.

Field of the Invention

The present invention relates to modified forms of pullulanase which maintain the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond, compositions which comprise the modified pullulanase, methods of making the modified pullulanase and methods of using the modified pullulanase, especially for the saccharification of starch.

Background of the Invention

Starch, the essential constituents of which are linear amylose and branched amylopectin glucose polymers can be converted into simple sugars by an enzymatic process carried out in two stages: one stage of liquefaction of the starch and one stage of saccharification of the liquefied starch. In order to obtain a high conversion level of the starch, pullulanase (E.C. 3.2.1.41, α-dextrin 6-glucano-hydrolase also termed alpha-1,6-glucosidase) has been used to catalyse the hydrolysis of alpha-1,6-glucosidic bonds.

Pullulanase enzymes in the art include those known to have optimum activity at acidic pH as well as those known to have activity at alkaline pH. Pullulanases described in the art include pullulanase derived from a strain of *Bacillus acidopullulyticus* described as having an optimum activity at a pH of 4-5 at 60 ° C (United States Patent No. 4,560,651); pullulanase derived from *Bacillus naganoensis* described as having a maximum activity at a pH of about 5, measured at 60 ° C and a maximum activity at a temperature of about 62.5 ° C, measured at a pH of 4.5 (United States Patent No. 5,055,403); pullulanase derived from *Bacillus sectorramus* described as having an optimum pH at 5.0 to 5.5 and an optimum temperature at 50 ° C (United States Patent No. 4,902,622); and pullulanase derived from *Bacillus brevis* PL-1 described as having activity at 4.5-5.5 at 60 ° C (JP 04/023985).

Pullulanase can be used with glucoamylase or ß-amylase for the production of high glucose and high maltose syrups. In addition to increasing the yields of sugars, pullulanase reduces reaction time, allows high substrate concentrations and a reduction of up to 50% in the use of glucoamylase (Bakshi et al. (1992) Biotechnology Letters vol.14 pp.689-694).

Summary of the Invention

The present invention relates to the surprising and unexpected discovery by Applicants that modified forms of pullulanase retain the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The present invention provides modified forms of pullulanase and methods for producing the modified pullulanase, especially in recombinant host microorganisms. The present invention further relates to enzymatic compositions comprising a modified form of pullulanase useful in the saccharification of starch and methods for the saccharification of starch comprising the use of the enzymatic compositions.

The present invention is based, in part, upon the discovery that when pullulanase obtained from Bacillus deramificans was recombinantly expressed and cultured in Bacillus licheniformis, the pullulanase produced was a mixture of modified forms yet the modified forms of pullulanase surprisingly retained the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The modified forms comprised B.deramificans pullulanase truncated at the amino terminus, i.e., having a deletion of amino acids from the amino terminus, and B.deramificans having additional amino acids at the amino terminus of the mature pullulanase. Therefore, in one aspect, the present invention provides modified pullulanase having a deletion of amino acids from the amino terminus of a pullulanase obtainable from a gram-positive or a gram-negative microorganism as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. In another aspect, the present invention provides modified pullulanase having additional amino acids at the amino terminus of a pullulanase obtainable from a gram-negative or gram positive microorganism as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The present invention also encompasses amino acid variations of a pullulanase obtainable from a gram-negative or gram positive microorganism as long as the

modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond.

In one embodiment, the modified pullulanase is a modification of pullulanase obtainable from Klebsiella species. In another embodiment, the modified pullulanase is a modification of pullulanase obtainable from Bacillus species. In yet another embodiment, the modified pullulanase is a modification of pullulanase obtainable from Bacillus including but not limited to *B.subtilis, B. deramificans, B. stearothermophilus, B. naganoensis, B. flavocaldarius, B. acidopullulyticus, Bacillus sp APC-9603, B. sectorramus, B. cereus, B. fermus.* In a preferred embodiment, the modified pullulanase is a modification of pullulanase obtainable from *B. deramificans* having the designation T89.117D (LMG P-13056) deposited June 21, 1993 under the Budapest Treaty in the LMG culture collection, University of Ghent, Laboratory of Microbiology, K.L. Ledeganckstraat 35, B-9000 GHENT, Belgium.

In one embodiment, the modified pullulanase has a deletion of about 100 amino acids from the amino terminus of a pullulanase. In another embodiment, the modified pullulanase has a deletion of about 200 amino acids from the amino terminus of a pullulanase and in yet another embodiment, the modified pullulanase has a deletion of about 300 amino acids from the amino terminus of a pullulanase.

In a further embodiment, the modified pullulanase has a deletion of 98 amino acids from the amino terminus of pullulanase obtainable from B. deramificans. In an additional embodiment, the modified pullulanase has a deletion of about 102 amino acids from the amino terminus of pullulanase obtainable from B. deramificans. In a further embodiment, the modified pullulanase has at least one additional amino acid at the amino terminus of pullulanase obtainable from B. deramificans. In another embodiment, the modified pullulanase has an additional amino acid residue, Alanine, added to the amino terminus of pullulanase obtainable from B.deramificans.

Modified forms of pullulanase having a decrease in molecular weight provide the advantage of higher specific activity (activity/unit weight) and therefore, less weight of pullulanase activity is required in a saccharification process to obtain results equivalent to the use of a naturally occurring pullulanase obtainable from or produced by a microorganism. The recombinant production of modified pullulanase as taught herein provides for enzymatic compositions comprising at least 60% and at least 80% pullulanase activity. In one embodiment, the enzymatic composition

comprises at least one modified pullulanase. In another embodiment, the enzymatic composition comprises more than one modified pullulanase. Such enzymatic compositions are advantageous to the starch processing industry due to their ability to produce a high glucose yield over a shortened saccharification time without the loss of glucose yield associated with reversion reaction products. Furthermore, it was unexpectedly found that in using an enzymatic composition comprising 20% glucoamylase and 80% pullulanase, higher starting dissolved solids (DS) could be used in a saccharification process, thereby increasing production plant capacity without an increase in capital investment. Additionally, saccharification at higher dissolved solids increases mechanical compression capacity thereby providing for a more energy efficient process.

In one embodiment, the present invention provides modified pullulanase produced by the method comprising the steps of obtaining a recombinant host cell comprising nucleic acid encoding mature pullulanase, culturing said host cell under conditions suitable for the production of modified pullulanase and optionally recovering the modified pullulanase. In one embodiment, the host cell is Bacillus, including but not limited to *B.subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis*. In a preferred embodiment, the Bacillus cell is B. licheniformis which comprises a first gene encoding the Carlsberg protease and a second gene encoding endo Glu C, the first and/or second gene which codes for the protease(s) having been altered in the Bacillus species such that the protease activity is essentially eliminated and the nucleic acid encoding the mature pullulanase is obtainable from B. deramificans.

In an alternative embodiment, the present invention provides methods for the production of a modified pullulanase in a recombinant host cell comprising the steps of obtaining a recombinant microorganism comprising nucleic acid encoding a modified pullulanase, culturing the microorganism under conditions suitable for the production of the modified pullulanase and optionally recovering the modified pullulanase produced. In one embodiment, the host cell is a gram-negative or gram-positive microorganism. In another embodiment, the host cell is a Bacillus including but not limited to *B.subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans,*

B. lautus and Bacillus thuringiensis. In another embodiment, the Bacillus cell is B. licheniformis which comprises a first gene encoding the Carlsberg protease and a second gene encoding endo Glu C, the first and/or second gene which codes for the protease(s) having been altered in the Bacillus species such that the protease activity is essentially eliminated and the nucleic acid encodes a modified pullulanase that is a modification of pullulanase obtainable from B.deramificans.

The present invention also provides nucleic acid comprising a polynucleotide sequence encoding modified pullulanase. In one embodiment, the nucleic acid has at least 70% identity, at least 80% identity, at least 90% identity or at least 95% identity to the polynucleotide sequence shown in SEQ ID NO: 1, which encodes pullulanase obtainable from B. deramificans. The present invention also provides expression vectors and host microorganisms comprising nucleic acid encoding a modified pullulanase of the present invention.

The present invention provides an enzymatic composition comprising at least one modified pullulanase of the present invention. In one embodiment, the enzymatic composition comprises multiple modified pullulanase forms. In another embodiment, the composition further comprises an enzyme selected from the group consisting of glucoamylase, alpha-amylase, beta-amylase, alpha-glucosidase, isoamylase, cyclomaltodextrin, glucotransferase, beta-glucanase, glucose isomerase, saccharifying enzymes, and/or enzymes which cleave glucosidic bonds. In a preferred embodiment, the enzymatic composition comprises a modified pullulanase and glucoamylase. In one embodiment, the glucoamylase is derived from an Aspergillus strain. In another embodiment, the glucoamylase is derived from an Aspergillus strain including but not limited to Aspergillus niger, Aspergillus awamori and Aspergillus foetidus. The enzymatic composition may be in a solid form or a liquid form. In one embodiment of the present invention, the enzymatic composition comprises at least 60% modified pullulanase and in another embodiment, the composition comprises at least 80% modified pullulanase.

The present invention also provides a process for the saccharification of starch, wherein said process allows for reduced concentrations of saccharification reversion by-products, comprising the step of contacting aqueous liquified starch with an enzyme composition comprising modified pullulanase. In one embodiment, the process further comprises the steps of heating said liquified starch, and

recovering product. In one embodiment of the process, the enzyme composition further comprises glucoamylase. In another embodiment of the process, the contacting is at a pH of about less than or equal to 7.0 and greater than or equal to 3 and in yet another, the pH is about 4.2. In a further embodiment of the process said heating is at a temperature range of between 55 and 65 degrees C. In another embodiment, the temperature is about 60 degrees C.

Brief Description of the Drawings

Figures 1A-1E illustrate the nucleic acid (SEQ ID NO:1) encoding the mature amino acid (SEQ ID NO:2) sequence of pullulanase obtainable from B. deramificans.

Figures 2A-2D are an alignment of amino acid sequences of pullulanase obtainable from B. deramificans (designated pullseqsig.seq.PRO), B. subtilis (designated subpull.seq.pro), and K. pneumonia (designated klebpnseqsig.seq.pro) showing the conserved domains and variability of the amino terminus of these pullulanases. This alignment also includes the signal sequences for the respective pullulanases.

Figures 3A-3C illustrate a timecourse of fermentation and the various species of modified pullulanase that are formed during the fermentation. Peak 1 designates the mature B. deramificans pullulanase having a molecular weight of 105 kD; peak 2 designates the modified pullulanase which has a deletion of 102 amino acids from the amino terminus of mature B. deramificans pullulanase; and peak 3 designates the modified pullulanase which has a deletion of 98 amino acids from the amino terminus as measured by standard HPLC analysis. Figure 3A illustrates the fermentation over 37 hours. Figure 3B illustrates the fermentation over 60 hours. Figure 3C illustrates the fermentation over 70 hours.

Figures 4A-4D illustrate the stability of the modified pullulanase species as a function of pH as measured by standard HPLC analysis. Figure 4A illustrates the pullulanase stability at 24 hours at a pH of 4.5 at room temperature. Figure 4B illustrates the pullulanase stability at 24 hours at a pH of 5.5 at room temperature. Figure 4C illustrates the pullulanase stability at 24 hours at a pH of 6.5 at room temperature. Figure 4D illustrates the pullulanase stability at 96 hours at a pH of 4.5 at room temperature.

Figures 5A-5C illustrate the effect of enzymatic compositions comprising various pullulanase and glucoamylase concentrations on the final glucose yield and disaccharide formation over saccharification time. The solid line refers to an enzymatic blend comprising 80% pullulanase activity (including modified pullulanase

having a deletion of 98 amino acids from the amino terminus of B. deramificans; modified pullulanase having a deletion of 102 amino acids from the amino terminus of B. deramificans; mature B. deramificans pullulanase and mature B. deramificans pullulanase having an additional amino acid (alanine) on the amino terminus) and 20% glucoamylase (20:80). The dotted line refers to an enzymatic composition comprising an enzyme blend comprising 75% glucoamylase obtainable from Aspergillus sp. and 25% mature pullulanase obtainable from B. deramificans (75:25). The solid line with squares refers to di-saccharides formed with the enzyme blend comprising 20% glucoamylase and 80% pullulanase activity as described above (20:80) over the saccharification time and the dotted line with circles refers to the di-saccharides formed with the 75:25 over the saccharification time. The left X-axis is % glucose yield and the right X-axis is % di-saccharides. Figure 5A refers to the saccharification process using 0.550 liters of enzymatic composition per metric ton of dissolved solids; Figure 5B refers to the saccharification process using 0.635 liters of enzymatic composition per metric ton of dissolved solids; Figure 5C refers to the saccharification process using 0.718 liters of enzymatic composition per metric ton of dissolved solids. This figure illustrates that a 20:80 enzymatic composition is able to increase the final glucose yield without an increase in undesirable disaccharide formation.

Figure 6 illustrates the effect of dissolved solids (%w/w) (Y axis) on the final glucose yield during saccharification of liquefied starch using enzyme compositions 20:80, 75:25, and 100 % glucoamylase at 0.55 liters of enzyme per metric ton of dissolved solids. Line A is the enzymatic composition 20:80 described in Figures 5A-5C; line B is the enzymatic composition 75:25 and line C is an enzymatic composition comprising 100% glucoamylase.

Detailed Description

Definitions

The term pullulanase as used herein refers to any enzyme having the ability to cleave the alpha-1,6 glucoside bond in starch to produce straight chain amyloses. These enzymes are preferably classified in EC 3.2.1.41 and include neopullulanases.

As shown in Figure 2, there are regions of similarity among pullulanases obtainable from gram positive and gram negative microorganisms. The amino acid alignment of pullulanase obtainable from Bacillus deramificans with pullulanase obtainable from K. pneumonia and B. subtilis reveals that when the conserved

domains are aligned, the amino terminus not associated with the conserved domains is of varying length. As used herein, the term "modified" when referring to pullulanase means a pullulanase enzyme in which the conserved domains are retained while any length of amino acids in the amino terminus portion of the naturally occurring amino acid sequence not associated with the conserved domains has been altered by a deletion of these amino acid residues or by addition of at least one amino acid to the amino terminus as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The deletion in the amino terminal amino acids of a pullulanase can be of varying length, but is at least three amino acids in length and the deletion can go no further than the beginning of the first conserved domain which in B. deramificans is the tyrosine at amino acid residue 310 as shown in Figure 1. In one embodiment, the deletion is about 100 amino acids from the amino terminus of the mature pullulanase. In another embodiment, the deletion is about 200 amino acids from the amino terminus of the mature pullulanase and in another embodiment, the deletion is about 300 amino acids from the amino terminus of the mature pullulanase. In a preferred embodiment, the modification is a deletion of 98 amino acids from the amino terminus of B. deramificans. In yet another embodiment, the deletion is 102 amino acids from the amino terminus of B. deramificans. In a further embodiment, the modification is an addition of at least one amino acid to the amino terminus of the naturally occurring mature pullulanase obtainable from Bacillus deramificans. In another preferred embodiment, the amino acid residue, Alanine, is added to the amino terminus of the mature pullulanase. As used herein the term "mature" refers to a protein which includes the N-terminal amino acid residue found after the natural cleavage site of the signal sequence.

As illustrated in Figures 2A-2D, B. deramificans pullulanase and K. pneumonia pullulanase are examples of pullulanases having similarities in the length of the amino terminus up to the beginning of the first conserved domain (which in B.deramificans is amino acid residue 310 Tyrosine). B. subtilis pullulanase is an example of a pullulanase having a shorter length of amino acid residues up to the beginning of the first conserved domain as shown in Figure 2B.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid" refers to peptide or protein sequences or portions thereof. The present invention

encompasses polynucleotides having at least 70%, at least 80%, at least 90% and at least 95% identity to the polynucleotide encoding *B. deramificans* pullulanase, as well as polynucleotides encoding a pullulanase activity capable of hybridizing to nucleic acid encoding *B. deramificans* pullulanase under conditions of high stringency.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

Detailed Description of the Preferred Embodiments

The present invention relates to the discovery that pullulanase recombinantly produced in a Bacillus host is modified yet unexpectedly retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The modification of the pullulanase product recombinantly produced appears to be a result of misprocessing of the signal sequence by a signal peptidase as well as susceptibility to extracellular proteolytic processing. The modified pullulanase is used to produce compositions and methods useful in the starch industry.

I. Pullulanase Sequences

The present invention encompasses any modified pullulanase which retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. A variety of pullulanases have been described in the art, including those obtainable from or naturally produced by gram-positive microorganisms as well as gram-negative microorganisms. Microorganisms which naturally produce pullulanase include, but are not limited to, *B.deramificans* (having the designation T89.117D in the LMG culture collection, University of Ghent, Laboratory of Microbiology-K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium) the nucleic acid (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence being disclosed in Figures 1A-1E; *B.naganoensis* (American Type Culture Collection, ATCC accession number 53909), disclosed in United States Patent No. 5,056,403 issued October 8, 1991; *B. acidopullulyticus* (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, NCIB 11607, NCIB 11610, NCIB 11611, NCIB 11636, NCIB 11637, NCIB 11639, NCIB 11638, NCIB 11647, NCIB 11777), disclosed in United States Patent No. 4,560,651, issued December 24, 1985; *B. sectorramus*

(Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Yatabe-machi, Tsukuba-gun, Ibaraki 305 Japan FERM BP-1471), disclosed in United States Patent No. 4,902,622, issued February 20, 1998; *Bacillus* FERM BP-4204 disclosed in United States Patent No. 5,387,516 issued February 7, 1995; *B. stearothermophilus* (SWISS-PROT id NEPU_BACST ac P38940); *B. cereus* var. *mycoides* (IFO 300) described in Y.Takasaki et al., 1976, Agric. Biol. Chem. 40:1515; *B. fermus* (IFO 3330); *Klebsiella pneumonia*, United States Patent No. 3,897,305 (SWISS-PROT id PULA_KLEPN ac P07206 and ATCC 15050; *Klebsiella aerogenes* (SWISS-PROT id PULA_KLEAE ac P07811); *Thermoanaerobium brockii* (ATCC No. 33075), United States Patent No. 4,628,028; *Streptomyces sp.* described in M. Yagisawa et al., 1972, J. Ferment. Technolo. 50:572; Caldicellulosiruptor saccharolyticus disclosed in Albertson et al., 1997, Biochimica et Biophysica Acta 1354:35-39; *Eschericia intermedia* Ueda et al., 1967, Applied Microbiology vol 15:492 United States Patent No. 3,716,455 (issued 1973) *Streptococcus mites* Walker 1968, Biochem. J., vol. 108:33; *Streptomyces* (Ueda et al., 1971, J. Ferment. Tech. Vol. 49: 552); *Flavochromogenes,* as described in United States Patent No. 4,902,622; *Flavobacterium esteromaticum* Japanese Patent Application Kokoku 18826/1973; *Cytophaga* United States Patent No. 3,790,446 issued 1974; *Lactobacillus*, *Micrococcus*, *Nocardia*, *Staphylococcus*, *Azotobactger*, *Sarcina* England patent 11260418, United States Patent No. 3,827940 issued 1974; and *Actinomycetes* United States Patent No. 3,741,873 issued 1973. Any pullulanase known in the art which comprises the conserved pullulanase regions as shown in Figures 2A-2D can be modified to have deletions or additions to the amino terminus as long as the modified pullulanase retains the ability to catalyse the hydrolysis of an alpha-1,6-glucosidic bond.

A nucleic acid sequence encoding a pullulanase can be obtained from a microorganism through hybridization technology using the nucleic acid sequences that encode the conserved domains of pullulanases (as shown in Figure 2) as primers and/or probes. (United States Patent 5,514,576; Southern, E. 1979, Methods Enzymol. 68:152-176; Saiki, et al. 1988, Science 239:487-491). In one embodiment disclosed herein for *B.deramificans* pullulanase, the naturally occurring nucleic acid (SEQ ID NO:1) encoding a mature pullulanase was introduced into *B.licheniformis* having a deletion of the Carlsburg protease (Jacobs et al., 1985, Nucleic Acid

Research 13:8913-8926) and endoGluC proteases (Kakudo et al., 1992, Journal of Bio. Chem. Vol. 267:23782-23788), the B.licheniformis comprising the nucleic acid encoding the mature pullulanase was cultured under conditions suitable for expression of said nucleic acid and secretion of the expressed pullulanase. The protease deletions in B.licheniformis were made through techniques known to those of skill in the art. Through the fermentation process, the expressed pullulanase was cleaved extracellularly into multiple pullulanase species which retain the ability to catalyse the hydrolysis of alpha-1,6-glucosidic bonds. The multiple species are a pullulanase having a deletion of the first 98 amino acid residues from the amino terminus and starting at glutamic acid, a pullulanase having a deletion of the first 102 amino acid residues from the amino terminus (and starting at glutamic acid), and a pullulanase having the addition of at least one amino acid residue to the amino terminus of the mature pullulanase, along with the mature pullulanase as shown in Figures 1A-1E. As shown in Example II, it appears that the extracellular cleaving into multiple species may be due to a protease activity in the fermentation broth.

In an alternate embodiment of the present invention, nucleic acid encoding a mature pullulanase is genetically engineered to create a modified pullulanase having a deletion of amino acids at the amino terminus or having amino acids added at the amino terminus. The genetically engineered pullulanase is introduced into a host cell, preferably a Bacillus host cell, and cultured under conditions suitable for expression and secretion of the modified pullulanase. Nucleic acid encoding a mature pullulanase can be a naturally occurring sequence, a variant form of the nucleic acid or from any source, whether natural, synthetic, or recombinant.

Regional sequence homologies in starch degrading enzymes have been disclosed in Janse et al. (1993) Curr. Genet. 24:400-407. Janse disclose the conserved regions in α-amylases that are implicated in substrate binding, catalysis, and calcium binding. An amino acid alignment of B.deramificans, B.subtilis and K. pneumonia pullulanases is shown in Figure 2.

When homologies were compared in starch degrading enzymes by Janse et al., four conserved regions where noted, Regions 1, 2, 3, and 4. Three of these regions were associated with specific functions found in starch-degrading enzymes: region 1: DVVINH (SEQ ID NO:5); region 2: GFRLDAAKH (SEQ ID NO:6); and region 4: FVDVHD (SEQ ID NO:7). Further analysis of five Type I pullulanase sequences by Albertson et al.(1997, Biochimica et

Biophysica Acta 1354:35-39) revealed other conserved regions among a group of gram-positive and gram-negative pullulanases. These include regions called DPY, A, B, C, D, E, and YNWGY. (SEQ ID NO:8) Two regions, DPY and YNWGY were identified as being characteristic of true pullulanases. Conserved regions A-E align closely with β-sheet elements as defined for amylases. In addition, two other conserved regions closer to the N-terminus of the pullulanase, referred to as Y and VWAP (SEQ ID NO:9) in Figures 2A-2D, indicate the limits of amino acid truncations in the N-terminal of pullulanases in general. This prediction is based on the lack of further conserved regions of identity among the known pullulanases beyond the Y region as one proceeds to the N-terminus. Due to the size heterogeneity of the known pullulanases, the N-terminal regions beyond the Y region call vary between approximately 100-300 amino acids. For the B. deramificans pullulanase, a truncation of 309 residues would leave the first conserved region (Y at amino acid residue 310 in Figures 1A-1E) intact.

B. deramificans pullulanase

Mature *B. deramificans* pullulanase comprises the amino acid sequence (SEQ ID NO: 2) shown in Figures 1A-1E. The following description of characteristics refers to mature *B. deramificans* pullulanase. *B. deramificans* pullulanase has an isoelectric point of between 4.1 and 4.5, is heat stable and active in a wide temperature range. The *B. deramificans* pullulanase is active at an acid pH. This pullulanase is capable of catalyzing the hydrolysis of α-1, 6-glucosidic bonds present both in amylopectin and in pullulan. It breaks down pullulan into maltrotriose and amylopectin into amylose. The polysaccharide pullulan, which is a polymer of maltotriose units connected to each other by alpha-1,6-linkages can be obtained from Aureobasidium pullulans (Pullaria pullulans) by the procedure of Ueda et al., Applied Microbiology, 11, 211-215 1963).

B. deramificans pullulanase hydrolyses amylopectin to form oligosaccharides (maltooligosaccharides). During this hydrolysis, the formation of oligosaccharides made up of about 13 glucose units (degree of polymerization of 13, this molecule is also called "chain A") is observed, followed by the formation of oligosaccharides made up of about 47 glucose units (degree of polymerization of 47, this molecule is also called "chain B').

The oligosaccharides with chains A and B are defined with reference to D. J. MANNERS ("Structural Analysis of Starch components by Debranching Enzymes" in "New Approaches to research on Cereal Carbohydrates", Amsterdam, 1985, pages 45-54) and B. E. ENEVOLDSEN ("Aspects of the fine structure of starch" in "New Approaches to research on Cereal Carbohydrates", Amsterdam, 1985, pages 55-60).

The B. deramificans pullulanase hydrolyses potato amylopectin. This hydrolysis can be carried out with an aqueous suspension of amylopectin in the presence of the pullulanase under the conditions of optimum activity of the pullulanase, that is to say at a temperature of about 60 °C and at a pH of about 4.3.

The B. deramificans pullulanase catalyses the condensation reaction of maltose to form tetraholosides (oligosaccharides having 4 glucose units).

The B. deramificans pullulanase has a half-life of about 55 hours, measured at a temperature of about 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate.

Half-life means that the pullulanase shows a relative enzymatic activity of at least 50%, measured after an incubation of 55 hours at a temperature of about 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate.

The B. deramificans pullulanase is heat stable at an acid pH and shows a relative enzymatic activity of at least 55%, measured after an incubation of 40 hours at a temperature of 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate. It shows a relative enzymatic activity of at least 70%, measured after an incubation of 24 hours under these same conditions.

Relative enzymatic activity means the ratio between the enzymatic activity measured in the course of a test carried out under the given pH, temperature, substrate and duration conditions, and the maximum enzymatic activity measured in the course of this same test, the enzymatic activity being measured starting from the hydrolysis of pullulane and the maximum enzymatic activity being fixed arbitrarily at the value of 100.

The B. deramificans pullulanase is furthermore stable in a wide range of acid pH values. Under the conditions described below, it is active at a pH greater than or equal to 3. In fact, the B. deramificans pullulanase shows a relative enzymatic activity of at least 85%, measured after an incubation of 60 minutes at a temperature

of about 60 °C in the absence of substrate and in a pH range greater than or equal to about 3.5.

Under the conditions described below, it is active at a pH of less than or equal to 7. In fact, the B. deramificans pullulanase shows a relative enzymatic activity of at least 85%, measured after an incubation of 60 minutes at a temperature of about 60 °C in the absence of substrate and in a pH range less than or equal to about 5.8.

It preferably shows a relative enzymatic activity of greater than 90%, measured in a pH range of between about 3.8 and about 5 under these same conditions.

The B. deramificans pullulanase develops an optimum enzymatic activity, measured at a temperature of about 60 °C, in a pH range greater than 4.0. It develops an optimum enzymatic activity, measured at a temperature of about 60 °C, in a pH range less than 4.8. The B. deramificans pullulanase preferably develops an optimum enzymatic activity, measured at a temperature of about 60 °C, at a pH of about 4.3. Furthermore, it develops an optimum enzymatic activity, measured at a pH of about 4.3, in a temperature range of between 55 and 65 °C, and more particularly at 60 °C.

The B. deramificans pullulanase develops an enzymatic activity of more than 80% of the maximum enzymatic activity (the maximum enzymatic activity being measured at a temperature of 60 °C and at a pH of 4.3) in a pH range between about 3.8 and about 4.9 at a temperature of about 60 °C.

The strain *Bacillus deramificans* T 89.117D has been deposited in the collection called BELGIAN CORRDINATED COLLECTIONS OF MICROORGANISM (LMG culture collection, University of Ghent, Laboratory of Microbiology - K. L. Ledeganckstraat 35, B - 9000 GHENT, Belgium) in accordance with the Treaty of Budapest under number LMG P-13056 on 21 June 1993.

II. Expression Systems

The present invention provides host cells, expression methods and systems for the production and secretion of modified pullulanase in gram-positive microorganisms and gram-negative microorganisms. In one embodiment, a host cell is genetically engineered to comprise nucleic acid encoding a modified pullulanase.

In another embodiment, the host cell is genetically engineered to comprise nucleic acid encoding a full length or mature pullulanase, which upon culturing produces a modified pullulanase. In a preferred embodiment, the host cell is a member of the genus Bacillus which has been modified to have a mutation or deletion of endogenous proteases.

Inactivation of a protease in a host cell

Producing an expression host cell incapable of producing a naturally occurring protease necessitates the replacement and/or inactivation of the naturally occurring gene from the genome of the host cell. In a preferred embodiment, the mutation is a non-reverting mutation.

One method for mutating nucleic acid encoding a protease is to clone the nucleic acid or part thereof, modify the nucleic acid by site directed mutagenesis and reintroduce the mutated nucleic acid into the cell on a plasmid. By homologous recombination, the mutated gene may be introduced into the chromosome. In the parent host cell, the result is that the naturally occurring nucleic acid and the mutated nucleic acid are located in tandem on the chromosome. After a second recombination, the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene for progeny of the parent host cell.

Another method for inactivating the protease proteolytic activity is through deleting the chromosomal gene copy. In a preferred embodiment, the entire gene is deleted, the deletion occurring in such as way as to make reversion impossible. In another preferred embodiment, a partial deletion is produced, provided that the nucleic acid sequence left in the chromosome is too short for homologous recombination with a plasmid encoded metallo-protease gene. In another preferred embodiment, nucleic acid encoding the catalytic amino acid residues are deleted.

Deletion of the naturally occurring microorganism protease can be carried out as follows. A protease gene including its 5' and 3' regions is isolated and inserted into a cloning vector. The coding region of the protease gene is deleted from the vector *in vitro*, leaving behind a sufficient amount of the 5' and 3' flanking sequences to provide for homologous recombination with the naturally occurring gene in the parent host cell. The vector is then transformed into the host cell. The vector integrates into the chromosome via homologous recombination in the flanking regions. This method leads to a strain in which the protease gene has been deleted.

The vector used in an integration method is preferably a plasmid. A selectable marker may be included to allow for ease of identification of desired recombinant microorgansims. Additionally, as will be appreciated by one of skill in the art, the vector is preferably one which can be selectively integrated into the chromosome. This can be achieved by introducing an inducible origin of replication, for example, a temperature sensitive origin into the plasmid. By growing the transformants at a temperature to which the origin of replication is sensitive, the replication function of the plasmid is inactivated, thereby providing a means for selection of chromosomal integrants. Integrants may be selected for growth at high temperatures in the presence of the selectable marker, such as an antibiotic. Integration mechanisms are described in WO 88/06623.

Integration by the Campbell-type mechanism can take place in the 5' flanking region of the protease gene, resulting in a protease positive strain carrying the entire plasmid vector in the chromosome in the pullulanase locus. Since illegitimate recombination will give different results it will be necessary to determine whether the complete gene has been deleted, such as through nucleic acid sequencing or restriction maps.

Another method of inactivating the naturally occurring protease gene is to mutagenize the chromosomal gene copy by transforming a microorganism with oligonucleotides which are mutagenic. Alternatively, the chromosomal protease gene can be replaced with a mutant gene by homologous recombination.

The present invention encompasses Bacillus host cells having protease deletions or mutations, such as deletions or mutations in apr, npr, epr, mpr, isp and/or bpf and/or others known to those of skill in the art. Disclosure relating to deleting protease(s) in the gram-positive microorganism, Bacillus, can be found in United States Patent Application Nos. 5,264,366; 5,585,253; 5,620,880 and European Patent No. EP 0369 817 B1

One assay for the detection of mutants involves growing the *Bacillus* host cell on medium containing a protease substrate and measuring the appearance or lack thereof, of a zone of clearing or halo around the colonies. Host cells which have an inactive protease will exhibit little or no halo around the colonies.

III. Production of modified pullulanase

For production of modified pullulanase in a host cell, an expression vector comprising at least one copy of nucleic acid encoding a modified pullulanase, and preferably comprising multiple copies, is transformed into the host cell under

conditions suitable for expression of the modified pullulanase. In accordance with the present invention, polynucleotides which encode a modified pullulanase, or fusion proteins or polynucleotide homolog sequences that encode amino acid variants of modified pullulanase (as long as the variant retains the ability to catalyse the hydrolysis of a α-1, 6 - glucosidic bond), may be used to generate recombinant DNA molecules that direct their expression in host cells. A host cell may be a gram-positive or a gram-negative cell. In one embodiment, the host cell belongs to the genus *Bacillus*. In another embodiment, the Bacillus host cell includes *B.subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis.* In a preferred embodiment, the gram positive host cell is *Bacillus licheniformis.*

As will be understood by those of skill in the art, it may be advantageous to produce polynucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular gram-positive host cell (Murray E et al (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

Altered pullulanase polynucleotide sequences which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally equivalent modified pullulanase. As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring modified pullulanase.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The encoded protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent modified pullulanase. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the variant retains the ability to modulate secretion. For example, negatively charged amino acids include

aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine, phenylalanine, and tyrosine.

The polynucleotides encoding a modified pullulanase of the present invention may be engineered in order to modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference, for example.

In one embodiment of the present invention, a polynucleotide encoding a modified pullulanase may be ligated to a heterologous sequence to encode a fusion protein. A fusion protein may also be engineered to contain a cleavage site located between the modified pullulanase nucleotide sequence and the heterologous protein sequence, so that the modified pullulanase may be cleaved and purified away from the heterologous fusion partner.

IV. Vector Sequences

Expression vectors used in expressing the pullulanases of the present invention in host microorganisms comprise at least one promoter associated with a modified pullulanase, which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the wild-type promoter for the selected pullulanase and in another embodiment of the present invention, the promoter is heterologous to the pullulanase, but still functional in the host cell. In one embodiment of the present invention, nucleic acid encoding the modified pullulanase is stably integrated into the microorganism genome.

In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also comprises one or more selectable markers. As used herein, the term selectable marker refers to a gene capable of expression in the host microorganism which allows for ease of selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to antibiotics, such as, erythromycin, actinomycin, chloramphenicol and tetracycline.

V. Transformation

A variety of host cells can be used for the production of modified pullulanase including bacterial, fungal, mammalian and insects cells. General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using DEAE-Dextran and electroporation. Plant transformation methods are taught in Rodriquez (WO 95/14099, published 26 May 1995).

In a preferred embodiment, the host cell is a gram-positive microorganism and in another preferred embodiment, the host cell is *Bacillus*. In a further preferred embodiment the Bacillus host is *Bacillus licheniformis*. In one embodiment of the present invention, nucleic acid encoding a modified pullulanase of the present invention is introduced into a host cell via an expression vector capable of replicating within the *Bacillus* host cell. Suitable replicating plasmids for *Bacillus* are described in Molecular Biological Methods for *Bacillus*, Ed. Harwood and Cutting, John Wiley & Sons, 1990, hereby expressly incorporated by reference; see chapter 3 on plasmids. Suitable replicating plasmids for *B. subtilis* are listed on page 92.

In another embodiment, nucleic acid encoding a modified pullulanase of the present invention is stably integrated into the host microorganism genome. Preferred host cells are gram-positive host cells. Another preferred host is *Bacillus*. Bacillus host cells include *B.subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis*. A preferred host is *Bacillus subtilis*. Another preferred host is *B. licheniformis*. Several strategies have been described in the literature for the direct cloning of DNA in *Bacillus*. Plasmid marker rescue transformation involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente et al., Plasmid 2:555-571 (1979); Haima et al., Mol. Gen. Genet. 223:185-191 (1990); Weinrauch et al., J. Bacteriol. 154(3):1077-1087 (1983); and Weinrauch et al., J. Bacteriol. 169(3):1205-1211 (1987)). The incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

Transformation by protoplast transformation is described for *B. subtilis* in Chang and Cohen, (1979) Mol. Gen. Genet 168:111-115; for B.megaterium in Vorobjeva et al., (1980) FEMS Microbiol. Letters 7:261-263; for B. amyloliquefaciens in Smith et al., (1986) Appl. and Env. Microbiol. 51:634; for B.thuringiensis in Fisher et al., (1981) Arch. Microbiol. 139:213-217; for B.sphaericus in McDonald (1984) J. Gen. Microbiol. 130:203; and B.larvae in Bakhiet et al., (1985, Appl. Environ.

Microbiol. 49:577). Mann et al., (1986, Current Microbiol. 13:131-135) report on transformation of *Bacillus* protoplasts and Holubova, (1985) Folia Microbiol. 30:97) disclose methods for introducing DNA into protoplasts using DNA containing liposomes.

VI. Identification of Transformants

Whether a host cell has been transformed with a modified or a naturally occurring gene encoding a pullulanase activity, detection of the presence/absence of marker gene expression can suggest whether the gene of interest is present However, its expression should be confirmed. For example, if the nucleic acid encoding a modified pullulanase is inserted within a marker gene sequence, recombinant cells containing the insert can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with nucleic acid encoding the pullulanase under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the pullulanase as well.

Alternatively, host cells which contain the coding sequence for a modified pullulanase and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

The presence of the pullulanase polynucleotide sequence in a host microorganism can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of the pullulanase polynucleotide sequences.

VII. Assay of Pullulanase Activity

There are various assays known to those of skill in the art for detecting and measuring pullulanase activity. An enzymatic unit of B. deramificans pullulanase (PUN) is defined as the amount of enzyme which, at a pH of 4.5, at a temperature of 60 degrees C and in the presence of pullulane, catalyses the release of reducing sugars at a rate of 1 μM glucose equivalent per minute.

Pullulanase activity can be measured in the presence or the absence of substrate. In one aspect, pullulanase activity can be measured in the presence of

substrate according to the following protocol. 1 ml of a 1% strength solution of pullulane in a 50 nM acetate buffer at pH 4.5 is incubated at 60 °C for 10 minutes. 0.1 ml of a solution of pullulanase corresponding to an activity of between 0.2 and 1 PUN/ml is added thereto. The reaction is stopped after 15 minutes by addition of 0.4 ml of 0.5 M NaOH. The reducing sugars released are analyzed by the method of SOMOGYI-NELSON [J. Biol. Chem., 153 (1944) pages 375-380; and J. Biol. Chem., 160 (1945), pages 61-68].

Another method can be used to analyze the pullulanase. The enzymatic reaction in the presence of pullulane is carried out in accordance with the above test conditions, and is then stopped by addition of sulphuric acid (0.1 N). The hydrolysis products of pullulane are then subjected to HPLC chromatography (HPX-87H column from BIO-RAD; the mobile phase is 10 mM H_2SO_4) in order to separate the various constituents. The amount of Maltotriose formed is estimated by measurement of the area of the peak obtained.

The so-called debranching activity, that is to say the hydrolysis of the α-1, 6-glucosidic bonds present in amylopectin, can be quantified by the increase in the blue coloration caused, in the presence of iodine, by the release of amylose from amylopectin. The debranching enzymatic activity is measured in accordance with the following protocol. 0.4 ml of a 1% strength amylopectin solution containing a 50 mM acetate buffer at pH 4.5 is incubated at 60 °C for 10 minutes. The reaction is initiated by addition of 0.2 ml of pullulanase, and is stopped after 30 minutes by addition of 0.4 ml of 0.3 M HC1. 0.8 ml of a 0.0025% (v/v) strength solution of iodine is then added to 0.2 ml of this reaction mixture and the optical density is measured at 565 nm.

A preferred method is disclosed in Example IV and relies on a colorimetric method that utilizes a soluble red-pullulan substrate for the determination of pullulanase activity. As the pullulanase enzyme hydrolyzes the substrate, soluble fragments of the dyed substrate are released into the reaction solution. The substrate is then precipitated with an ethanol solution and the supernatant is evaluated for color intensity with spectrophotometer. In this assay, the degree of color intensity is proportional to the enzyme activity.

VIII. Secretion of Recombinant Proteins

Means for determining the levels of secretion of a modified pullulanase in a host microorganism and detecting secreted proteins include, using either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, a Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al (1983, J Exp Med 158:1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting specific polynucleotide sequences include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the nucleotide sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in US Patent No. 4,816,567 and incorporated herein by reference.

IX. Purification of Proteins

Host cells transformed with polynucleotide sequences encoding modified pullulanase may be cultured under conditions suitable for the expression and recovery of the pullulanase from cell culture. The protein produced by a recombinant gram-positive host cell comprising a mutation or deletion of endogenous protease activity will be secreted into the culture media. Other recombinant constructions may join the modified pullulanase polynucleotide sequences to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53).

Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein can be used to facilitate purification.

X. Uses of The Present Invention

Modified pullulanase

A modified pullulanase of the present invention finds use in various industries including the food industry, the pharmaceuticals industry and the chemical industry. A modified pullulanase can be used in baking as an "anti-staling" agent, that is to say as an additive to prevent bread becoming stale during storage, or in brewing during production of low-calorie beers. The pullulanase can also be used in the preparation of low-calorie foods in which amylose is used as a substitute for fats. The pullulanase can be used, for example, to clarify fruit juices.

For food applications, the pullulanase can be immobilized on a support. The techniques for immobilization of enzymes are well known to the expert.

The pullulanase can also be used to hydrolyse amylopectin and to form oligosaccharides starting from this amylopectin. The pullulanase can also be used to form tetraholosides starting from maltose.

The pullulanase can also be used to condense mono- or oligo-saccharides, creating bonds of the alpha-1, 6 type. The pullulanase can be used for liquefaction of starch.

A modified pullulanase can be used in the same manner as its respective unmodified form. A modified pullulanase, which in unmodified form has activity under alkaline conditions, will retain activity under alkaline conditions. A modified pullulanase which in unmodified form has activity under acidic conditions, will retain activity under acidic conditions. A particular modified pullulanase will be formulated according to the intended uses. Stabilizers or preservatives can also be added to the enzymatic compositions comprising a modified pullulanase. For example, a modified pullulanase can be stabilized by addition of propylene glycol, ethylene

glycol, glycerol, starch, pullulane, a sugar, such as glucose or sorbitol, a salt, such as sodium chloride, calcium chloride, potassium sorbate, and sodium benzoate, or a mixture of two or more of these products. The enzymatic compositions according to the invention can also comprise one or more other enzymes. Such enzymes include but are not limited to glucoamylase, alpha-amylase, beta-amylase, alpha-glucosidase, isoamylase, cyclomaltodextrin, glucotransferase, beta-glucanase, glucose isomerase, saccharifying enzymes, and enzymes which cleave glucosidic bonds or a mixture of two or more of these. In a preferred embodiment, the enzymatic composition comprises a modified pullulanase of the present invention at 80% and a glucoamylase at 20%.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto. All references and patent publications disclosed herein are hereby incorporated by reference.

EXAMPLES

Example I:

Example I illustrates the production of a modified pullulanase as described herein. The nucleic acid sequence encoding a pullulanase is modified by recombinant DNA techniques such as standard PCR primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. (Saiki, R. K., et al., 1988, Science 239:487-491.) and PCR fusion techniques (Fleming, A. B., et al. *Appl. Environ. Microbiol.* **61**, 3775-3780). DNA encoding the desired modified pullulanase is fused to the C-terminus of a signal sequence, preferably a host microorganism signal sequence. This construct is cloned and transformed into a host cell, such as, B. subtilis or B.licheniformis, and cultured under standard fermentation conditions. The modified pullulanase is purified from the fermentation broth and assayed for activity.

Example II

Example II describes the modified forms of pullulanase obtained upon culturing the recombinant B. licheniformis host cell comprising nucleic acid encoding a mature B.deramificans pullulanase wherein the host cell has a deletion of the Carlsburg and endo GluC proteases. The B. licheniformis was cultured under

standard fermentation conditions in a complex media. The fermentation broth was subjected to standard HPCL analysis and the results are shown in Figure 3A-3C which illustrate a timecourse of the various species of modified pullulanase formed during the fermentation process. Peak 1 designates the mature B. deramificans pullulanase having a molecular weight of 105 kD; peak 2 designates the modified pullulanase which has deletion of 102 amino acids from the amino terminus of mature B. deramificans pullulanase; and peak 3 designates the modified pullulanase which has a deletion of 98 amino acids from the amino terminus as measured by standard HPLC analysis. The modified pullulanase species which has an additional amino acid on the mature sequence is not detectable by HPLC analysis but was detected upon nucleic acid sequencing. Figures 3A-3C illustrate that over fermentation time, Peak 1 corresponding to the mature B. deramificans pullulanase decreases while Peaks 2 and 3 increase. Figures 4A-4D illustrate the stability of the modified pullulanase produced upon fermentation of B.licheniformis having a deletion of the Carlsburg and endoGluC proteases. B. licheniformis comprising nucleic acid encoding a mature B. deramificans was cultured under conditions suitable for the expression and secretion of the modified pullulanase and the fermentation broth was adjusted to a pH of 4.5, 5.5 and 6.5 at room temperature. The modified pullulanase was most stable at a pH of 4.5.

Example III

Example III describes the saccharification process comparing enzymatic compositions comprising different percentages of pullulanase. Enzymatic compositions comprising either 20% glucoamylase:80%modified pullulanase (20:80) activity or 75% glucoamylase:25% pullulanase activity (75:25) were tested in saccharification processes at a concentration of 0.550, 0.635 and 0.718 liters of enzymatic composition per metric ton of dissolved solids. As shown in Figures 5A-5C, an enzymatic composition comprising 20% glucoamylase and 80% pullulanase activity is able to increase the final glucose yield without an increase in undesirable disaccharide formation. Furthermore, the absolute concentration of the 20:80 enzyme composition can be increased without the undesirable increase in disaccharide formation that is seen with the 75:25 enzyme composition or glucoamylase alone.

Example IV

Example IV describes an assay for the determination of activity of a modified pullulanase of the present invention. This assay is based on a colorimetric method that utilizes a soluble red-pullulan substrate for the determination of pullulanase activity.

Reagent Preparation

A 200mM Sodium Acetate buffer pH 5.0 w/Acarbose (density~1.010) was prepared by weighing out 16.406g of anhydrous Sodium acetate or 27.21g of Sodium acetate trihydrate and dissolving it in 900 mls of deionized water (DI) in 1 L graduated cylinder by stirring with a magnetic stir bar. The pH was adjusted to 5.0 with glacial acetic acid. 0.300g of Acarbose was added to the solution and allowed to dissolve. The volume was brought up to 1000mL with DI water and mixed.

2% Red Pullulan Substrate Preparation

1.00g of Red Pullulan substrate was weighed out and dissolved in 50 mL of sodium acetate buffer by stirring with a magnetic stir bar for approximately 20-30 minutes. This solution is stable for two weeks stored at 4°C.

Preparation of a working standard

Using positive displacement pipettes a 1:10 dilution of the Pullulanase Standard was prepared. The assigned activity of the standard was 195.9 ASPU/ml.
The following working concentrations were prepared from the standard from the 1:10 stock dilution.

Sample Preparation

For a control, Optimax L-300 MA7EC191 PU B1 3-19A available from Genencor International was used. The control was diluted 1:1000 in sodium acetate buffer. All samples were diluted in sodium acetate buffer to obtain final reaction absorbances that fall on the calibration curve. The sample was brought to room temperature. A minimum of 100ul of sample was used for the initial dilution.

Assay Procedure

250 ul of each standard working concentration, control and sample was placed into two appropriately labeled microcentrifuge tubes. To each tube 250 ul of 2% substrate solution was added with a repeater pipette and a 12.5 ml Combitip set on 1. The samples were Vortexed for 3 seconds and incubated at 40°C for 20 minutes.
The samples were remove from the water bath and immediately 1.0 ml of 95% EtOH was added to the samples in the same order as above. A repeater pipette and a 12.5

or 50 ml Combitip set on 4 or 1, was used. The samples were vortexed for 3 seconds. The samples were incubated at room temperature for 5-10 minutes, then centrifuged for ten minutes in a benchtop centrifuge. The supernatant of the standards and samples were read in a spectrophotometer at 510 nm using 1.5 mL cuvettes. (The spectrophotometer was zeroed with 95% EtOH)

Calculations

Using the standard concentrations and correlating absorbances (subtracting the blank absorbance), a calibration curve is developed with a computer spreadsheet, programmable calculator, or graph paper. The curve should be linear over the range of the standard concentrations with a correlation coefficient (r) of 0.998 or greater. The precision of the assay should fall between 5-10% CV. For liquids: u/ml = (u/ml from standard curve)* (sample dilution)